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# HUMAN EPIDERMAL TRANSAMIDASE*

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The possible presence of ε-(γ-glutamyl)lysine covalent bonds in human epidermal proteins prompted a study of transamidase activity in human hair-free epidermis. Callus contains an enzyme which catalyzes the incorporation of radioactive putrescine into α-casein. The enzyme is active without prior treatment with exogenous proteolytic enzymes. The putrescine incorporation is calcium dependent and inhibited by iodoacetamide. The enzyme was partially purified (50-fold over starting material), and has an apparent molecular weight between 50,000 daltons and 55,000 daltons by agarose 0.5m gel filtration. The apparent molecular weight is unaltered by chromatography in the presence of 11 mM $CaCl_2$, a condition known to dissociate plasma transglutaminase (Factor XIII) into its ultimate subunits. The enzyme is active over a wide pH range up to pH 10.4. The $K_m$ for putrescine varies by 1-fold over the pH range 6.0 to 10.2, although enzyme activity increases at least 20-fold over the same pH range. The human epidermal transamidase is similar to the guinea-pig hair follicle transglutaminase and cow snout transamidase in its ability to cross-link fibrin.

The structural proteins of keratinized tissues are stabilized by at least two kinds of covalent bonds: disulfide bonds and ε-(γ-glutamyl)lysine bonds. Both of these are post-translational covalent bonds formed after the ribosomal phase of protein synthesis. The structural role and importance of the disulfide bond in determining the chemical and physical properties of wool is well recognized and reviewed in [1]. The ε-(γ-glutamyl) dipeptide has been demonstrated conclusively in the internal root sheath of guinea-pig hairs [2,3] and the medullary tissue of porcupine quills [2,3].

The medullary proteins of hair and quill have marked resistance to chemical and proteolytic treatment and these properties may be related in part to the ε-(γ-glutamyl)lysine cross-links. The formation of the ε-(γ-glutamyl)lysine bond is catalyzed by specific transamidases named transglutaminases, and these enzymes have been demonstrated in the guinea-pig hair roots [4,5], sheep hair roots [5], in the epithelium of the cow snout [6], and in frog and turtle epidermis [6]. Only the enzyme of the guinea-pig hair root has been demonstrated to be a specific transglutaminase [4]. Since direct study of the receptor sites of proteins which are substrates for the epidermal enzymes have not been accomplished, the epidermal enzyme will be referred to as a transamidase.

In this paper we describe some of the properties and characteristics of transamidase activity in human hair-follicle-free epidermis.

## MATERIALS AND METHODS

Callus was obtained from normal subjects and individuals with hyperkeratotic soles. General body epidermis and sole epidermis were obtained from fresh surgical specimens or at the time of autopsy. Bio-Gel A-0.5m (200–400 mesh) was purchased from Bio-Rad, diethylaminoethyl (DEAE) cellulose (DE-52) and carboxymethyl-cellulose (CM-52) from H. Reeve Angel, α-casein from Worthington, dithiothreitol from Cyclo Chem. (Los Angeles, Calif.), thrombin from Parke-Davis, and fibrinogen from A. B. Kabi (Stockholm, Sweden). Other reagents and salts were the highest grade available. 1,4-$^3$H-Putrescine (182.8 Ci/mole), 1,4-$^{14}$C putrescine (19.5 Ci/mole), and Aquasol were purchased from New England Nuclear.

The standard transamidase assay was done by adding 10–100 λ of the preparation to be tested to 0.5 ml of a solution containing 0.1 M Tris-acetate (pH 7.5) with 1 mM EDTA, 10 mM $CaCl_2$, 0.60 mM putrescine hydrochloride (60.9 Ci/mole when 1,4-$^3$H-putrescine was used, and 6.5 Ci/mole when 1,4-$^{14}$C-putrescine was a substrate), and 3 mM dithiothreitol, with 3.3 mg α-casein. The mixture was incubated in a shaking water bath for 30 min at 37°C and the reaction stopped by the addition of 0.7 ml 10% TCA. Five milliliters of 5% TCA containing 0.1% unlabeled putrescine was then added and incubation at 37°C continued for another 30 min. The casein precipitate was collected on a Whatman GF/C filter, washed with 50 ml of cold 5% TCA with 0.1% putrescine, and the precipitated radioactivity counted in Aquasol with a Packard 3375 scintillation spectrometer. The basic assay measures the incorporation of radioactively labeled putrescine into acid-precipitable material and differs slightly

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* The following abbreviations are used: Tris for Tris (hydroxymethyl) amino methane, EDTA for ethylenediamine tetracetic acid, DTT for dithiothreitol, SDS for sodium dodecylsulfate, TCA for trichloroacetic acid, CMC for carboxymethyl-cellulose.

from that of Chung and Folk [4] by containing unlabeled putrescine in the wash solutions. With this addition reagent blanks had 100 to 150 cpm. Factor XIII-free fibrinogen was made by treating fibrinogen with 3.3 M urea [7].

pH was measured at 25°C with a combination electrode. Protein was measured by the technique of Lowry et al [8].

*Enzyme Preparation: Stratum Corneum*

Ten grams of palmar stratum corneum of one person was stirred with 100 ml of 0.25 M sucrose in 0.05 M Tris-chloride with 1 mM EDTA, pH 7.5, for 1½ hr at 4°C. All further procedures were at 4°C. After homogenization with a Tekmar homogenizer for 1 min, the mixture was centrifuged at 40,000 x *g* for 20 min and the supernatant centrifuged at 100,000 × *g* for 45 min. The supernatant was dialyzed against 4 liters of 0.01 M Tris-chloride, 1 mM EDTA, pH 7.5, overnite. A small precipitate from this solution was removed by centrifugation at 40,000 x *g* for 20 min. The supernatant was applied to a 0.9 × 15 cm DEAE column, washed with 100 ml of 0.01 M Tris-chloride, 1 mM EDTA, pH 7.5, and then eluted with a 200 ml linear gradient of NaCl (0–0.3 M) in the same buffer. A sharp peak of enzyme activity coincided with the start of the gradient. Elution with 0.5 M NaCl produced a second peak of activity accounting for 10% of the total applied activity. The peak tubes of the initial peak were concentrated on an Amicon UM-10 filter and applied to a 1.5 × 44 cm Agarose column equilibrated with 10 mM Tris-chloride, 1 mM EDTA, 0.15 M NaCl, pH 7.5, with a flow rate of 12 ml per hr. The tubes with activity were brought to pH 6.0 with 1 M acetic acid the dialyzed against 500 ml of 5 mM Tris-acetate, 1 mM EDTA (pH 6.0) for 3 hr and then applied to a 0.9 × 15 cm carboxymethyl cellulose column. The column was washed with 100 ml of the equilibrating buffer (0.05 M Tris-acetate, 1 mM EDTA, pH 6.0) and then eluted with a 200 ml linear gradient of NaCl (0–0.5 M) in the same buffer. The effluent was monitored continuously for protein. Enzyme activity coincided with the single protein peak after the start of the gradient.

In some experiments the active peak after agarose gel filtration was concentrated to 3 ml on an UM-10 membrane and reapplied to the agarose column which had been reequilibrated with the starting buffer plus 11 mM $CaCl_2$.

*Gel Electrophoresis*

The effluent after CMC chromatography was run in discontinuous electrophoresis at pH 9.5 [9] and also in SDS protein electrophoresis [10] with bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome C as molecular-weight markers. A parallel unstained gel from discontinuous electrophoresis was sliced into 1 mm wide segments, and eluted with 0.5 ml of a solution containing 0.1 M Tris-acetate, pH 7.5, 1 mM EDTA, 10 mM DTT, and 3.3 mg α-casein for 18 hr at 4°C. 0.6 mM putrescine (6.5 Ci/mole) was then added and the mixture incubated for 30 min at 37°C. The reaction was stopped with 10% TCA and activity determined as previously described.

*Enzyme Properties*

$K_m$s were obtained at six different substrate concentrations of putrescine using the enzyme preparation after CMC chromatography. Assays were started by the addition of enzyme to the reaction mixture. Calcium activation was measured in the enzyme preparation after CMC chromatography.

In experiments in which EDTA or iodoacetamide were tested as inhibitors, these reagents were added before the putrescine substrate.

In experiments to demonstrate cross-linking activity, 220 μg of Factor XIII-free fibrinogen were dissolved in 0.2 ml of a 0.1 M Tris-acetate (pH 8.0) buffer with 80 mM NaCl, 1 mM EDTA, 5 mM $CaCl_2$, and 1 mM DTT. One NIH unit of thrombin and 32 μg of semipurified enzyme (eluate from CMC chromatography) were added. Controls without enzyme, and without thrombin were also prepared. Incubation was performed for 3¾ hr at 37°C. The reaction was stopped and contents of the tubes were dissolved in an equal volume of 0.2 M phosphate buffer (pH 7.2) containing 10 M urea, 2% SDS, and 2% β-mercaptoethanol and incubated overnite at 37°C. Aliquots were applied to SDS acrylamide gels according to the procedure of Weber and Osborne [10].

## RESULTS

*Epidermal transamidases could be isolated from* hyperkeratotic soles, normal sole epidermis, or normal body epidermis.

Radioactive putrescine incorporation was proportional to the amount of enzyme used (Fig. 1) and to time of incubation. Enzyme activity was inhibited by EDTA and iodoacetamide. No pretreatment of the sample with exogenous proteolytic enzymes was necessary for enzyme activity.

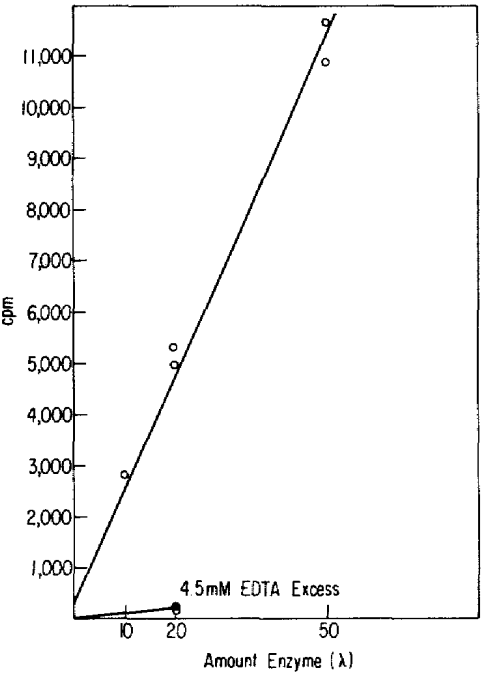


FIG. 1. Incorporation of $^{14}C$ putrescine into α-casein. Transamidase activity was measured by incubating 100,000 x *g* epidermal homogenates or column fractions with the standard incubation mixture at pH 7.5. Enzyme activity was proportional to the amount of homogenate added and dependent on free calcium ion in the reaction mixture. Zero time blanks were subtracted from assay results. Details of assay in text.

Transamidase activity was partially purified (Tab. I) using standard procedures, but loss of activity prevented more complete isolation of the active enzyme. There was one peak of enzyme activity with an apparent molecular weight between 50,000 and 55,000 daltons on an agarose 0.5m column (Fig. 2*A*).

Disc electrophoresis of the CMC effluent showed one major protein band with Commassie blue; however, when a parallel unstained gel was assayed for transamidase activity no enzyme activity was found in this zone or elsewhere on the gel. SDS electrophoresis of the CMC effluent showed four bands staining with Commassie blue—the most heavily stained had a molecular weight of 85,300; the other bands had apparent molecular weights of 55,000, 41,700, and 33,500.

DTT in crude preparations and in the most purified preparations increased enzyme activity by about 30%. Equal aliquots of the CMC effluent in the standard assay mixture at pH 7.5 without added DTT incorporated 529 cpm; with 3.6 mM DTT, 533 cpm; with 18 mM DTT, 699 cpm; and with 36 mM DTT, 678 cpm.

Increasing the pH of the reaction mixtures markedly increased transamidase activity (Fig. 3). The $K_m$ for putrescine over the pH range studied varied 1-fold while enzyme activity varied 25-fold. The $K_m$s in mM for putrescine were 4.0 at pH 6.1; 2.0 and 2.5 in two separate experiments at pH 7.5; 2.5 at pH 8.9; and 3.7 at pH 10.2. The increase in activity with increasing pH was present at substrate levels of $K_m$ and 1/10 of $K_m$. Calcium activation of the enzyme occurred completely with a 2.45 mM calcium excess to the amount of EDTA present (Tab. II). The putrescine incorporation at both pH 10.4 and 8.5 is dependent on calcium and free sulfhydryl groups (Tab. III). At pH 10.5, putrescine incorporation increases with increases in casein concentration (Tab. IV). At a lower pH, 8.46, the incorporation of putrescine into casein is inversely related to casein concentration.

There was no significant change in the relative elution volume of enzyme activity when the proteins with enzymatic activity from an agarose 0.5m column were rechromatographed on the identical column which had been reequilibrated with column buffer containing 11 mM $CaCl_2$ (Figs. 2*A* and *B*).

The purified enzyme cross-linked fibrin (Fig. 4). The $\alpha$ and $\gamma$ chains were decreased in amount and heavy molecular bands appeared at the top of the

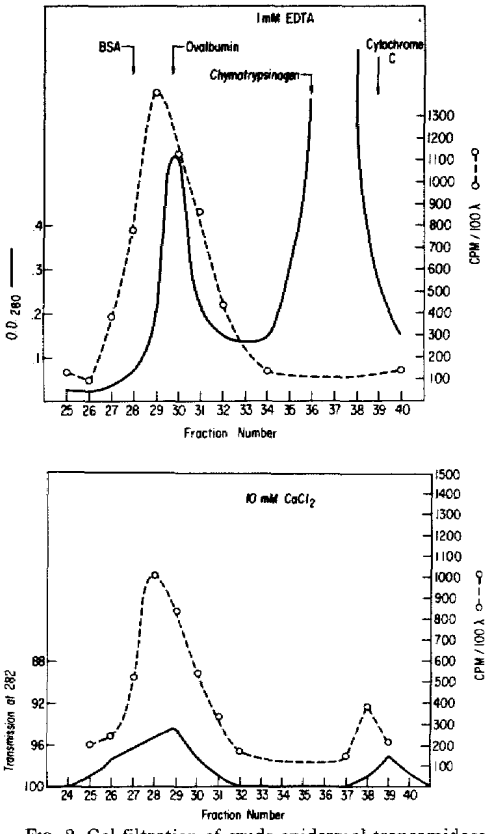


FIG. 2. Gel filtration of crude epidermal transamidase on agarose 0.5m. (*A*) On a 1.5 x 44 cm column of Bio-gel Agarose 0.5m a sample of epidermal transamidase was chromatographed in a 0.05 M Tris-acetate buffer (pH 7.5) with 1 mM EDTA and 150 mM NaCl. 1.6-ml fractions were collected. The elution volumes of molecular weight markers run on the same column were determined during continuous monitoring of the column effluent with an LKB uvicord II. *Dashed line* represents enzyme activity (*B*) The tubes with enzyme activity from the column in (*A*) were concentrated on a UM-10 filter and applied to the column which had now been equilibrated with the initial buffer plus 11 mM $CaCl_2$. Fractions of the same volume were collected with enzyme activity having the same elution profile. Seventy-six percent of the total activity applied to this column is recovered from the second column; 6% of the total activity is in tubes 37–39. No peak of absorbance appeared before tube 25 or after tube 40. 100 λ of the fractions were assayed at pH 7.5 as described in *Materials and Methods*.

TABLE I. *Summary of purification of human epidermal transamidase*

| | Protein (*mg*) | Total enzyme activity units[a] | Specific activity (*units/mg protein*) | Purification |
|---|---|---|---|---|
| Homogenate supernatant with dialysis | 276 | 20.82 | .075 | 1 |
| DEAE effluent | 11.9 | 11.73 | .99 | 13.2 |
| Agarose 0.5m peak | 2.6 | 8.03 | 3.09 | 41.2 |
| CMC effluent | .58 | 2.26 | 3.90 | 52.0 |

[a] Units = nmoles putrescine incorporated per 30 min at 37°C.

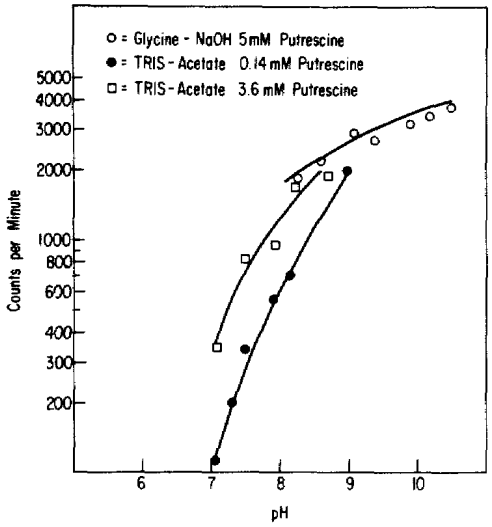


FIG. 3. pH activity curves of human epidermal transamidase. The same amount of an epidermal transamidase preparation was added to 0.5 ml of an assay mixture containing 3.3 mg $\alpha$-casein, 3 mM dithiothreitol, 1 mM EDTA, and 0.1 M of the above buffer with the above putrescine concentrations. The complete reaction was incubated at 37°C for 30 min, stopped with 10% TCA, and from that point treated as described in *Materials and Methods*.

TABLE II. *Calcium activation of epidermal transamidase*

Each assay tube contained 3.3 mg $\alpha$-casein with 60 mM Tris-acetate (pH 7.5), 0.60 mM EDTA, 3.0 mM dithiothreitol, 0.60 mM putrescine hydrochloride (60.9 Ci/mole), and the indicated amount of calcium in excess to the EDTA present. The reaction was started by adding the same amount of enzyme after CMC chromatography in 1 mM EDTA to each tube and incubating at 37°C for 30 min. After stopping the reaction with 0.7 ml of 10% trichloroacetic acid the assay was conducted as indicated in *Materials and Methods*.

| | cpm |
|---|---|
| Enzyme with 0.60 mM EDTA | 80 |
| with 2.45 mM $Ca^{++}$ excess | 1197 |
| with 5.5 mM $Ca^{++}$ excess | 1102 |
| with 8.55 mM $Ca^{++}$ excess | 1233 |
| with 11.6 mM $Ca^{++}$ excess | 765 |

gel ($a_n$). These have the mobility of $\alpha$ polymers. A band (**x**) which may present a $\gamma$ dimer or a $\alpha$-$\gamma$ dimer is also present.

## DISCUSSION

The human epidermal transamidase resembles the guinea-pig hair follicle transglutaminase [4,11] and the cow snout transamidase [6]. All three enzymes are inhibited by either iodoacetamide or EDTA, the enzymes have similar apparent molecular weights of between 50,000 and 60,000 on agarose gel filtration, they have similar elution behaviors on DEAE and CMC, and they cross-link fibrin. DTT, although not necessary for enzyme activity, increases activity by 20 to 30% in the human and cow snout transamidase properties. Buxman and Wuepper confirm the properties of cow snout transamidase [12].

Table V summarizes the epidermal and follicular transamidases described to date. The Harding and Rogers [5] studies differed from those of Chung and Folk [4] since the former authors assayed the esterase activity of the follicular enzymes and showed an inhibition of guinea-pig enzyme activity by anti-Factor XIII. The sheep hair follicle enzyme which is not inhibited by EDTA [5] was very different from all other transglutaminases, and confirming studies of its behavior would be of interest. Enzyme studies of follicular tissue have to be interpreted carefully for two reasons; first, these tissues are often contaminated with epidermis, and secondly, most preparations con-

TABLE III. *Metal and sulfhydryl dependence of putrescine incorporation*

In this assay equal amounts of the same enzyme preparation were added to a 0.52 ml volume of the assay mixture containing 3.3 mg $\alpha$-casein with 3.0 mM dithiothreitol, 0.60 mM putrescine hydrochloride (60.9 Ci/mole), 10 mM $CaCl_2$, and 100 mM glycine NaOH buffer at the indicated pH. Iodoacetamide, or excess EDTA, was added in the indicated assays to the enzyme preparation before the addition of putrescine. After incubation at 37°C for 30 min the reaction was stopped with 0.7 ml of 10% TCA and analyzed as indicated in *Materials and Methods*.

| | cpm | |
|---|---|---|
| | (pH 10.4) | (pH 8.5) |
| Standard conditions, pH 10.4 | 31,854 | 8344 |
| Standard conditions + 16.5 mM EDTA (5 mM EDTA excess) | 86.0 | 42.0 |
| Standard conditions + 35 mM iodoacetamide | 113.0 | — |

TABLE IV. *Effect of casein concentration at two different pHs on epidermal transamidase activity*

In this assay equal volumes of the same enzyme preparation were added to 0.5 ml containing the above amount of casein/ml, 5 mM putrescine, 1 mM EDTA, 3 mM dithiothreitol, and glycine-NaOH adjusted to pH 8.5 or 10.5 as indicated. The reaction mixture was incubated at 37°C for 30 min, the reaction stopped with 0.7 ml of 10% TCA, and then handled as described in the text.

| Final casein concentration (*mg/ml*) | cpm | |
|---|---|---|
| | pH 8.5 | pH 10.5 |
| 0.61 | 4545 | 13,310 |
| 1.22 | 3546 | 18,531 |
| 2.44 | 3445 | 22,713 |
| 3.66 | 2787 | 23,399 |

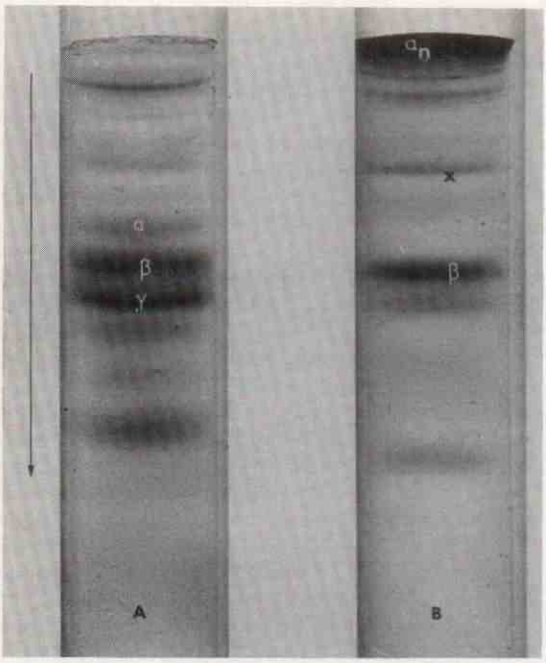


FIG. 4. Cross-linking of fibrin by semipurified epidermal transamidase. Details of the experiment are described in the text. *A* is a reaction mixture containing Factor XIII-free fibrinogen in buffer and 1 NIH unit thrombin; *B*, Factor XIII-free fibrinogen, thrombin, and 32 $\mu$g of epidermal transamidase. $\alpha$, $\beta$, $\gamma$ represent non-cross-linked fibrin chains. Bands with the mobility of $\alpha$-chain polymers are at the top of the gel; a new band **(x)** which may represent a $\gamma$ dimer or an $\alpha$-$\gamma$ dimer is present in the gel. The *arrow* represents the direction of electrophoretic migration.

tain both internal and external root sheaths. Furthermore, follicular tissue [4] has two transglutaminases, one with esterase activity which is similar to liver transglutaminase, and another transglutaminase similar to epidermal transamidase.

In order to obtain information on the possible presence of subunits in epidermal transamidase, gel filtration in the presence and absence of calcium was performed. When plasma Factor XIII was studied in a similar set of experiments, its molecular weight decreased to the size of its ultimate subunits with apparent molecular weights of 70,000 and 80,000 daltons [11]. Similar studies with the follicular or epidermal enzymes have not been previously reported. In our studies (Fig. 2) there is no evidence that this has happened; however, 24% of the applied enzyme activity was not recovered. Similar experiments starting with larger amounts of material may answer these questions of the ultimate size of the functional subunits. Chung and Folk [4] suggested a possible 27,000 subunit for guinea-pig follicular transglutaminase on the basis of gel electrophoresis in SDS, while their gel filtration and gel electrophoresis experiments with different percentages of acrylamide in the gels suggested a molecular weight of 54,000.

The pH activity curve for the epidermal enzyme is different from that for the liver enzyme [13] or plasma Factor XIII [14]. Liver enzyme has a $V_{max}$ for hydrolysis and hydroxylamine incorporation at about pH 6.0. Factor XIII has an overall $V_{max}$ at pH 7.5, but this represents an optimum level for

TABLE V. *Mammalian epidermal and hair follicle transamidases*

| Author: | *Chung et al [4, 11]* | *Harding and Rogers [5]* | | *Goldsmith et al [6]* |
|---|---|---|---|---|
| Tissues: | Guinea pig hair follicle | Rat and guinea pig hair follicle | Sheep hair follicle | Cow snout epidermis |
| Substrates: | $^{14}$C-Putrescine<br>$^{14}$C-Methylamine | $^{14}$C-Glycine ethyl ester | | $^{14}$C and $^{3}$H Putrescine<br>Dansylcadaverine |
| Metal requirements: | EDTA inhibits<br>Iodoacetate inhibits | EDTA inhibits<br>Iodoacetate inhibits | EDTA did not inhibit<br>Iodoacetic inhibits only in presence of calcium | EDTA inhibits<br>Iodoacetamide inhibits |
| Molecular weight: | 54,000 (gel filtration)<br>27,000 SDS electrophoresis<br>55,000 (varying concentrations acrylamide gels) | | | 50–60,000 (gel filtration) |
| Immunological identity: | No reaction or inhibition with antibody to liver transglutaminase | Antihuman Factor XIII inhibits | No cross-reaction with anti-Factor XIII | |

thrombin activation and amine incorporation. With the human epidermal transamidase, since the $K_m$ of the putrescine substrate does not change appreciability over the range studied and since studies at low putrescine and high putrescine concentration do not affect the rate considerably, we consider the effect of pH to be on the other substrate in the reaction, namely, casein. The high pHs may be allowing increased reactivity of casein's glutamines. At lower pHs, high concentrations of casein may be inhibitory by precipitating during the reaction due to their being directly cross-linked by the action of epidermal transamidase. At high pHs the effect of increasing the casein concentration is as would be expected with increasing substrate concentration. The effect of pH will have to be studied with better-defined lower-molecular-weight protein substrates.

Complete evaluation of the kinetic behavior of the enzyme will require more completely purified enzyme and a well-defined substrate with only one glutamine acceptor side such as the acetylated B-chain of oxidized insulin. Many of the kinetic effects observed in the present studies may be explained by heterogeneity of glutamine sites present in casein.

The persistance of the enzyme in an active form in stratum corneum and callus may be responsible for the lability of the enzyme during purification procedures, since the enzyme may have been subjected to some proteolytic attack by epidermal proteases in vivo. Exogenous proteolytic enzyme activation of the epidermal enzyme is not necessary for enzyme activity; however, there is no information on whether the enzyme exists in a proenzyme form which is activated in vivo or during isolation procedures. The gel filtration experiments with and without calcium suggest the enzyme activity is not related to Factor XIII which may be in the epidermis.

The role of this enzyme in human epidermal metabolism is moot until the epidermal proteins which may be participating in covalent cross-links are identified. Such studies are in progress.

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